Biochem. J. (1975) **149**, 169–177
Printed in Great Britain

A Method for Investigating the Effect of Temperature on the 695nm Band of Insoluble Cytochrome *c*

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(*Received* 11 *November* 1974)

A method is described for computer analysis of simple spectrophotometric changes in particulate systems, and this has been applied to the bleaching of the 695nm band of insoluble ferricytochrome *c* by temperature. The results show that insolubilization has no effect on the standard enthalpy change but lowers the value for the standard entropy change. This effect appears to be independent of the concentration of the gel matrix to which the cytochrome *c* is bound, but dependent on the ionic strength of the surrounding solution

In the last few years there has been much interest in proteins insolubilized on to inert carriers, as model systems for proteins bound in naturally occurring matrices (Silman & Katchalski, 1966; Brown *et al.*, 1968; Gestrelius *et al.*, 1972), as specific tools for analytical chemistry and the fast-expanding field of affinity chromatography (Guilbault, 1966; Goldstein & Katchalski, 1968; Kay, 1968). Other workers have used insolubilized enzymes for studying enzyme action or protein denaturation (Hornby *et al.*, 1968). In order to make the measurements necessary for such a work, a number of methods have been developed, including continuous-flow-through columns of insolubilized enzyme (Hornby *et al.*, 1966), or front-face fluorescence from flow-through spectrophotometer cells packed with insoluble enzyme (Gabel *et al.*, 1971).

In many cases, however, direct measurements by absorption spectrophotometry would be extremely valuable for comparison with those measurements for the soluble enzyme; but immense difficulties are encountered from gel scattering and absorbance. In this paper, a method for analysing direct spectrophotometric observations from suspensions of insoluble enzyme is described, and this has been used to define thermodynamic parameters associated with the disappearance of the 695nm band of insoluble ferricytochrome *c* in response to high temperatures.

Materials and Methods

Horse heart cytochrome *c* was supplied by Boehringer Corp. (London) Ltd., London W.5, U.K. Cytochrome *c* concentrations were determined spectrophotometrically by using the millimolar extinction coefficient of 27.6 for the reduced material at 550nm (Margoliash & Frohwirt, 1959). Agarose powder was obtained from Miles–Seravac (Pty.) Ltd. (Maidenhead, Berks., U.K.). All other chemicals were AnalaR grade, and all solutions were prepared in distilled water.

Cytochrome *c* was insolubilized on to agarose gel suspension by the method of Kay & Lilly (1970) by using 2-amino-4,6-dichloro-*s*-triazine. The suspensions were made by blending 1vol. of agarose gel of the desired concentration with 2vol. of distilled water, until the suspensions were able to pass through a no. 25 gauge Luer hypodermic needle.

Absorption spectrophotometry was performed on an SP.700A Unicam spectrophotometer or a split-beam spectrophotometer constructed according to the design of Yang & Legallais (1954), with the use of a Hilger and Watts Monospek 600 monochromator filter, and an Aminco Chance dual-wavelength/split-beam recording spectrophotometer.

The temperature experiments were carried out in a 1cm cell fitted with a water jacket. Water of the required temperature was pumped from a water bath through the jacket until the cytochrome *c* solution or suspension reached a steady temperature.

The results were analysed on an ICL 4-75 computer in the Edinburgh Multi-Access System.

Theory

There are three contributions to any spectrophotometric observation of a suspension of chromophore; these are (1) the chromophore absorbance;

(2) the scattering and absorbance of the matrix suspension; (3) the 'hiding' effect caused by high concentrations of chromophore in local and distinct packets, parts of which may have 0% transmission, effectively 'hiding' the chromophore behind it.

If three wavelengths are chosen (l, m and n) to observe a change centred at m, such that the scattering and absorbance contributions of the gel are the same at l, m and n, and such that the 'hiding' effect is also identical, then the following quantity can usefully be defined:

$$R_x = \frac{A_l - A_m}{A_m - A_n} \tag{1}$$

where A is the absorbance at a wavelength shown as a subscript. If the soluble and insoluble forms of the chromophore have identical extinction coefficients, then the values of R_x will be identical. For example if the absorbance is lowered by a 'hiding factor' x, and is increased by a gel-absorbance value of g then:

$$R_x = \frac{(xA_l + g) - (xA_m + g)}{(xA_m + g) - (xA_n + g)} = \frac{A_l - A_m}{A_m - A_n}$$

In the simple case, where a single spectrophotometric change takes place, the fractional change (y) can be defined so:

$$y = \frac{I - A}{I - F}$$

where I is the absorbance when $y = 0.0$, F is the absorbance when $y = 1.0$ and A is the absorbance when $0.0 < y < 1.0$, i.e.:

$$A = I - y(I - F) \tag{2}$$

Substituting eqn. (2) into eqn. (1), then:

$$R_x = \frac{[I_l - y(I_l - F)] - [I_m - y(I_m - F_m)]}{[I_m - y(I_m - F_m)] - [I_n - y(I_n - F_n)]} \tag{3}$$

or rearranging:

$$R_x = \frac{(I_l - I_m)\{1 - y[1 - (F_l - F_m)/(I - I_m)]\}}{(I_m - I_n)\{1 - y[1 - (F_m - F_n)/(I_m - I_n)]\}}$$

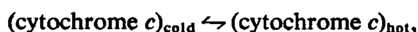
Substituting R_x^I for R_x when $y = 0.0$, a for $(F_l - F_m)/(I_l - I_m)$, and b for $(F_m - F_n)/(I_m - I_n)$, then:

$$R_x = R_x^I \left\{ \frac{[(1-y)/y] + a}{[(1-y)/y) + b} \right\} \tag{3A}$$

In this paper this equation has been applied to the change in absorbance of the 695 nm band of cytochrome *c* with temperature by making use of the equation:

$$1/K_{eq.} = (1 - y)/y = e^{\Delta G^0/RT} \tag{4}$$

where $K_{eq.}$ is the equilibrium constant for the reaction:



$$(\text{cytochrome } c)_{cold} \rightleftharpoons (\text{cytochrome } c)_{hot},$$

ΔG^0 is the standard free energy change, T is the absolute temperature and R is the gas constant. Substituting eqn. (4) in eqn. (3A) and expanding ΔG^0, then:

$$R_x = R_x^I \left\{ \frac{a + e^{\Delta H^0/RT - (\Delta S^0/R)}}{b + e^{\Delta H^0/RT - (\Delta S^0/R)}} \right\} \tag{5}$$

where ΔH^0 is the standard enthalpy change, and ΔS^0 is the standard entropy change. Practically, R_x and T are the only variables and thus a series of results for R_x and T were optimized by using a computer programmed for the Simplex Optimization Method of Nelder & Mead (1965). The initial guesses for a and b were estimated from graphs of R_x and A_λ against T, and those used for ΔH^0 and ΔS^0 were 60.0 kJ/mol and 0.17 kJ/mol per °K.

In a more complex system where two regions of different optical changes overlap, eqn. (5) cannot be used and a more complex equation has to be derived. In this case, the fractional saturations at $\lambda = l$, $\lambda = m$ and $\lambda = n$ are different (i.e. y_l, y_m and y_n), and at any wavelength:

$$A = [I - y(I - F)]_x + [I - y(I - F)]_Y$$

Substituting into eqn. (1) and rearranging as before:

$$R_x = \frac{(I_l - I_m)_x \cdot (a_x + e^{\Delta G_x^0/RT}) + (I_l - I_m)_Y \cdot (a_Y + e^{\Delta G_Y^0/RT})}{(I_m - I_n)_x \cdot (b_x + e^{\Delta G_x^0/RT}) + (I_m - I_n)_Y \cdot (b_Y + e^{\Delta G_Y^0/RT})}$$

Substituting k_1 for a_x, k_2 for $(I_l - I_m)/(I_l - I_m)$, k_3 for a_Y, k_4 for $(I_m - I_n)_x/(I_l - I_m)$, k_5 for b_x, k_6 for $(I_m - I_n)_Y/(I_l - I_m)$ and k_7 for b_y, then:

$$R_x = \frac{(k_1 + e^{\Delta G_x^0/RT}) + k_2(k_3 + e^{\Delta G_y^0/RT})}{k_4(k_5 + e^{\Delta G_x^0/RT}) + k_6(k_7 + e^{\Delta G_y^0/RT})} \tag{6}$$

As $\Delta G^0{}_x = \Delta H^0{}_x + T\Delta S^0{}_x$ and $\Delta G^0{}_Y = \Delta H^0{}_Y + T\Delta S^0{}_Y$, eqn. (6) has 11 unknowns and is thus impractical to use unless certain constants can be fixed, and the others can be estimated very accurately. Hence, although the method outlined for simple systems is extremely useful, it cannot be reasonably applied to more complex systems.

Computing

The computer was programmed to accept estimated or guessed values, for the constants which were progressively changed by the program. Each set of constants tried corresponded to a set of absorbances which were compared with the observed data by the sum of squared deviations (cost). The simplex contracted when a reflexion resulted in a cost which was worse than the second worst cost, and the program accepted this as a minimum when the mean-squared deviation of the cost of each vertex of the simplex, compared with that of the centroid, was less

than 1.0×10^{-8} for the first minimum found, and less than 1.0×10^{-9} for minima found after the first re-expansion. The best value for the minimum (i.e. the smallest cost) was stored as the parameter FA and compared with each minimum cost found [Y(L)] such that the programme was terminated when the criterion

$$\left|\frac{\mathrm{FA}-\mathrm{Y(L)}}{\mathrm{Y(L)}}\right|<5.0\times10^{-5}$$

was satisfied. The simplex was restarted by reflexion of the guessed values about the values at the first termination (first minimum). The second simplex was terminated by using the criteria described above, but in addition the 'error landscape' in the region of the second minimum was investigated by calculating the fractional increase in cost when each parameter in turn was increased by 5%.

The values for ΔH^0, ΔS^0, a and b for the first and second minima were substituted into eqn. (5), and the observed values of $\ln(R_x)$ were compared with the values of

$$\ln\left\{\frac{a+e^{[(\Delta H^0/RT)-(\Delta S^0/R)]}}{b+e^{[(\Delta H^0/RT)-(\Delta S^0/R)]}}\right\}$$

by using a straight-line regression analysis. The slope (compared with 1.0), the intercept [compared with $\ln(R_x^I)$] and the correlation coefficient were used as another check on the validity of the optimization.

Practically, the fractional increase in cost for a 5% change in parameter rarely exceeded 10 for a and b, but averaged at about 30 and 800 for ΔH^0 and ΔS^0 respectively.

The effect of the guessed values were investigated in the regions of the optimized parameter values for the soluble cytochrome c system (Table 1) and the insoluble cytochrome c system (Table 2). For each example a set of points was calculated and standard deviations of 0%, 5% and 10% were imposed. The simplex was started from two unfavourable starting

Table 1. *Results from the optimizations of eqn. (5) using data simulating the experimental results obtained from soluble cytochrome*

(*a*) Values used to calculate the theoretical points: (*b*) details of the input data for the optimizations and the standard deviation of the points; (*c*) results of the optimizations. The initial estimates were set as 10% above or 10% below the real answers or to those used for the experimental optimizations.

(*a*)

	ΔH^0 ($kJ\cdot mol^{-1}$)	ΔS^0 ($kJ\cdot mol^{-1}\cdot {}^\circ K^{-1}$)	a	b	R_x^I
Real answers	60.94	0.179	5.74	0.0857	0.32

(*b*)

Computer run	S.D. of points	Initial estimates ΔH^0	ΔS^0	a	b	No. of points
1	0.00	54.0	0.1611	5.166	0.0771	52
2	0.05	54.0	0.1611	5.166	0.0771	52
3	0.10	54.0	0.1611	5.166	0.0771	52
4	0.00	67.03	0.1969	6.314	0.0943	52
5	0.05	67.03	0.1969	6.314	0.0943	52
6	0.10	67.03	0.1969	6.314	0.0943	52
7	0.00	60.00	0.1800	5.400	0.10	52
8	0.05	60.00	0.1800	5.400	0.10	52
9	0.10	60.00	0.1800	5.400	0.10	52

(*c*)

Computer run ...	1	2	3	4	5	6	7	8	9
Minimum 1									
ΔH^0 ($kJ\cdot mol^{-1}$)	*	*	*	67.04	66.96	66.57	62.53	61.29	61.01
ΔS^0 ($kJ\cdot mol^{-1}\cdot {}^\circ K^{-1}$)	*	*	*	0.1987	0.1975	0.1975	0.1849	0.1802	0.1792
a	*	*	*	5.179	5.906	5.041	5.097	5.682	5.725
b	*	*	*	0.1469	0.1673	0.1385	0.0962	0.0904	0.0869
Minimum 2									
ΔH^0 ($kJ\cdot mol^{-1}$)	56.10	57.15	56.12	67.00	59.97	66.21	62.76	61.70	61.04
ΔS^0 ($kJ\cdot mol^{-1}\cdot {}^\circ K^{-1}$)	0.1595	0.1617	0.1525	0.1986	0.1685	0.1945	0.1857	0.1809	0.1792
a	9.66	31.33	22.78	5.234	14.13	6.379	5.053	6.063	5.815
b	0.0286	0.1393	0.0260	0.1487	1.1755	0.1707	0.0988	0.1023	0.08821

* Optimizations that failed because of either a time limit set to stop the simplex when it was working very slowly, or because one or more parameters had reached values of less than 1.0×10^{-5}.

Table 2. *Results from the optimizations of eqn.* (5) *using data simulating the experimental results obtained from insoluble cytochrome c*

(*a*) Values used to calculate the theoretical points; (*b*) details of the input data for the optimizations and the standard deviations of the points; (*c*) results of the optimizations. The initial estimates were set as 10% above or 10% below the real answers or to those used for the experimental optimizations.

(*a*)

	ΔH^0 ($kJ \cdot mol^{-1}$)	ΔS^0 ($kJ \cdot mol^{-1} \cdot {}^\circ K^{-1}$)	a	b	R_x^l
Real answers	60.20	0.154	9.75	0.154	1.3

(*b*)

Computer run	S.D. of points	Initial estimates				No. of points
		ΔH^0	ΔS^0	a	b	
1	0.00	54.18	0.1386	8.775	0.0680	70
2	0.05	54.18	0.1386	8.775	0.0680	70
3	0.10	54.18	0.1386	8.775	0.0680	70
4	0.00	66.22	0.1694	10.73	0.0832	70
5	0.05	66.22	0.1694	10.73	0.0832	70
6	0.10	66.22	0.1694	10.73	0.0832	70
7	0.00	60.00	0.1800	5.40	0.10	70
8	0.05	60.00	0.1800	5.40	0.10	70
9	0.10	60.00	0.1800	5.40	0.10	70

(*c*)

Computer run ...	1	2	3	4	5	6	7	8	9
Minimum 1									
ΔH^0 ($kJ \cdot mol^{-1}$)	59.97	60.68	59.18	63.46	61.06	62.14	60.76	60.33	60.39
ΔS^0 ($kJ \cdot mol^{-1} \cdot {}^\circ K^{-1}$)	0.1461	0.1466	0.1433	0.1719	0.1629	0.1716	0.1614	0.1600	0.1616
a	23.43	36.85	24.15	4.053	4.687	2.574	4.987	5.122	4.309
b	0.2714	0.9132	0.0015	0.2957	0.1364	0.1717	0.1116	0.1114	0.1199
Minimum 2									
ΔH^0 ($kJ \cdot mol^{-1}$)	60.50	61.42	58.95	60.07	59.43	59.29	59.08	59.92	60.64
ΔS^0 ($kJ \cdot mol^{-1} \cdot {}^\circ K^{-1}$)	0.1437	0.1436	0.1386	0.1512	0.1526	0.1535	0.1532	0.1515	0.1642
a	37.50	56.49	39.37	12.99	8.645	7.304	7.141	12.23	3.478
b	0.4357	2.6622	0.0019	0.0499	0.0585	0.0001	0.0019	0.2665	0.1245

points (i.e. all the parameters +10% or −10% of the real values) or from the starting point used in the experiments below.

It can be seen that the optimized values for ΔH^0 and ΔS^0 are more consistent than those for a and b, and that the optimized values in Table 1 are more sensitive to the starting point than those in Table 2. It is clear also that superimposed standard deviations of up to 10% have little effect on the optimized parameters. Both Tables indicate that the starting points (used for the experimental analysis) give optimized values of ΔH^0 and ΔS^0 within 5% of the real values.

Results

Ferricytochrome *c* insolubilized on to agarose gel showed a distinct 695 nm band, but slightly diminished in size in comparison with that for soluble ferricytochrome *c* even when oxidized by ferricyanide (see Fig. 1). The decrease in size was thought to be a feature of insoluble ferricytochrome *c* rather than a result of slight polymerization during the insolubilization process, as no spectroscopic evidence was obtained to show that any carbonmonoxy-ferrocytochrome *c* was formed at pH 7.4 in the presence of dithionite and saturated CO solution.

Eqn. (5) was used to find ΔH^0 and ΔS^0 for the temperature-sensitive 695 nm band of insoluble cytochrome *c*. It is a convenient system to use, as Schejter & George (1964) have measured ΔH^0 and ΔS^0 for the soluble protein by using spectrophotometric observations at 695 nm only, and thus two methods can be compared directly.

For this system, m was equal to 695 nm, and l and n were chosen such that $n-m = m-l$ ($=\Delta\lambda$). The optimized values for ΔH^0 and ΔS^0 were selected as 'true' minima when (1) the cost was favourable and (2) the regressed slope and intercept for $\ln(R_x)$ observed versus:

$$\ln\left[\frac{a+e^{\Delta G^0/RT}}{b+e^{\Delta G^0/RT}}\right]$$

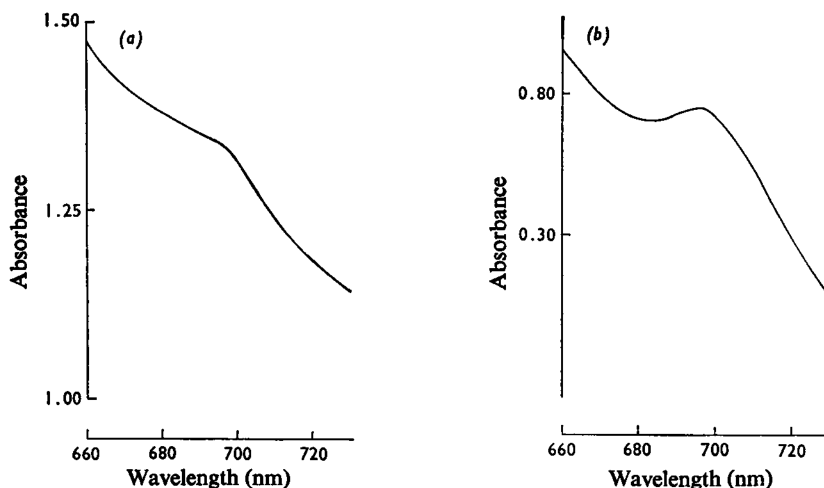


Fig. 1. *695nm bands of (a) ferricytochrome c insolubilized on to 6% agarose gel (at 12°C) and (b) ferricytochrome c in solution (at 9°C)*

Both spectra were obtained from suspensions or solutions in distilled water. The potassium ferricyanide used to oxidize the cytochrome *c* was removed on a column of Sephadex G-25 for soluble cytochrome *c* or by washing and centrifugation for the insoluble cytochrome *c* suspension.

Table 3. *Optimized values of ΔH^0 and ΔS^0 for soluble cytochrome c*

The experimental results were obtained by the method described in the text. The cytochrome *c* solution was prepared in distilled water. Values for the successful first and reflected optimizations are given.

No. of experimental points	ΔH^0 (kJ·mol^{-1})	ΔS^0 (kJ·mol^{-1}·°K^{-1})	$\Delta\lambda$(nm)
13	60.94	0.1794	35
13	59.48	0.1809	30
	60.80	0.1847	
13	58.17	0.1796	25
	67.80	0.2130	
13	58.20	0.1833	15
	63.06	0.2062	
52	58.20	0.1798	Pooled
	57.91	0.1786	results

were approximately equal to 1.0 and $\ln(R_X^I)$ respectively. A series of different starting points on two or three different sets of results for the same experimental system gave fairly consistent optimized values, and any movement of the simplex away from these values invariably resulted in curve fittings which made $e^{\Delta G/RT}$ equal to a value outside the limits set in the program (i.e. +20 and −20), and this automatically substituted an artificially high cost. This accuracy is demonstrated by the agreements between the optimized values before and after reflexions shown in the results. Practically, bad results usually led to very erroneous values for the regressed slope and intercept. No optimization was accepted which resulted in (1) a value of *a*, *b*, ΔH^0 or ΔS^0 less than 1.0×10^{-5} or (2) a fractional change of cost from a 5% increase in the optimized value of ΔH^0 and ΔS^0 of less than 10 or (3) a slope or correlation coefficient not satisfying the criteria $0.9 < \text{slope} < 1.1$, and correlation coefficient >0.9. The rules of acceptance, outlined above, were applied to all results given in this paper.

There was a sigmoidal decrease in absorbance of ferricytochrome *c* solutions at each of the wavelengths investigated, and ΔG^0 and ΔS^0, calculated from the 695nm absorbance by the method of Schejter & George (1964), were found to be 60.05kJ·mol^{-1} and 0.182kJ·mol^{-1}·°K^{-1} respectively, in agreement both with the values of Schejter & George (1964) and the optimized values shown in Table 3. A comparison of the variances for the sets of observations at each $\Delta\lambda$, with the variance of the pooled results, indicated that they were part of the same distribution with a certainty of greater than 99%, and an optimization using all the results can also be seen in Table 3.

The experiments with insoluble cytochrome *c* were carried out with cytochrome *c* insolubilized on to suspensions of 2, 4 and 6% agarose gels at cytochrome *c* concentrations of 3.39, 4.34 and 5.49mg/ml respectively. The temperature profiles were similar

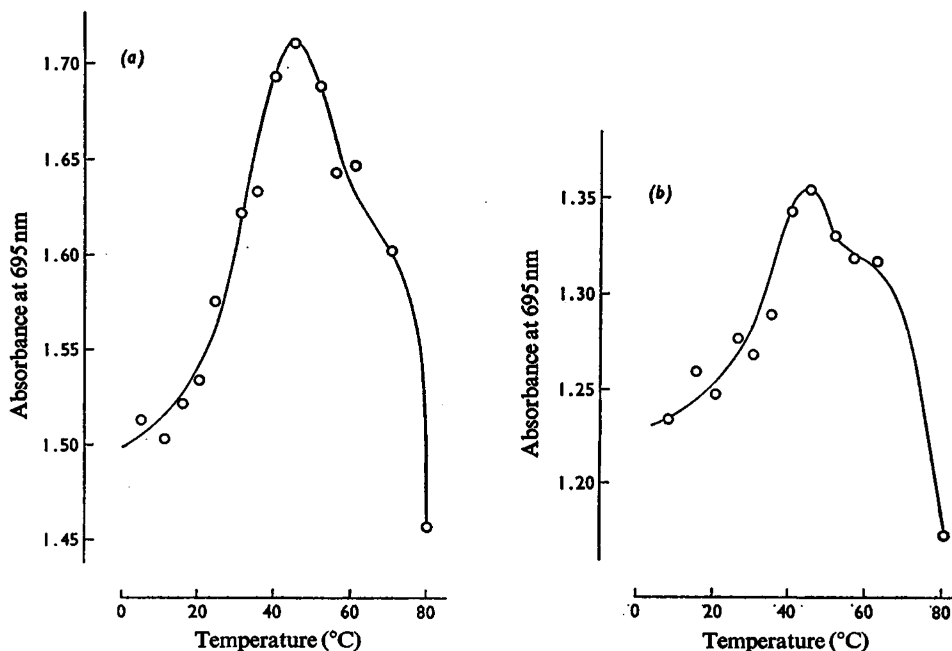


Fig. 2. *Temperature profiles of (a) cytochrome c insolubilized on to 6% agarose gel and (b) cytochrome c insolubilized on to 4% agarose gel*

All suspensions were prepared in distilled water and the wavelength used was 695nm. Cytochrome *c* concentrations were 5.49mg/ml for the 6% gel and 4.34mg/ml for the 4% gel.

at all wavelengths investigated but very different from those for soluble cytochrome *c* (see those for E_{695} shown in Fig. 2). A comparison of the profiles at 670, 695 and 730nm for cytochrome *c* insolubilized on to 2 and 6% gels can be seen in Fig. 3(*a*), which shows three regions of change, i.e. (1) a wavelength-independent change up to 43°C, (2) a decrease to a shoulder at approx. 70°C and (3) a sharp fall over 70°C. The temperature profile showed hysteresis, having a peak at 43°C during heating, and at 24°C during cooling from 80°C (see Fig. 3*b*). These results can be compared with the temperature profiles of (a) insoluble cytochrome *c* at 530nm (a region not associated with temperature sensitivity in soluble cytochrome *c*), which shows only the first two regions of change (see Fig. 4*a*), and (b) that for a very thick suspension of 2% agarose gel, which shows only a small sigmoidal decrease in absorbance in the 695nm region (see Fig. 4*b*). All these results are consistent with a change which in part is due to the agarose gel and indicated by the chromophore, and in part is due to a change in the chromophore. The former is most probably caused by breaking of gel interchain and intrachain hydrogen bonds, thus loosening and expanding the structure, and decreasing the 'hiding effect'. This is consistent with the enormous hysteresis curves shown by changes in optical rotation of agarose gel in response to heating and cooling, which themselves match the difference between liquefaction and setting temperatures (Rees, 1972).

Further evidence for this line of thought was obtained by repeating the heating experiment with cytochrome *c* insolubilized on to 2% agarose gel in the presence of 3M-NaCl. The results (Fig. 5) show a slow but steady decrease in the absorbance at 695nm with none of the complex changes of the previous experiments.

In all the experiments with insoluble cytochrome *c*, the 695nm band did not appear to be as sensitive to temperature as that of soluble cytochrome *c*, and this was confirmed by optimization, by using eqn. (5), of the results for cytochrome *c* insolubilized on to 2 and 6% gels. The analyses of variances for the insoluble cytochrome *c* results showed, as before, that the optimized results at values of $\Delta\lambda$ of 35, 30, 25, 20 and 15 were parts of the same distribution with a certainty of greater than 99%, and so all were pooled to give greater accuracy to the optimization. As can be seen from Table 4, ΔH^0 is the same for soluble and insoluble cytochrome *c* (±6.5%), but ΔS^0 is decreased on insolubilization by more than 20%,

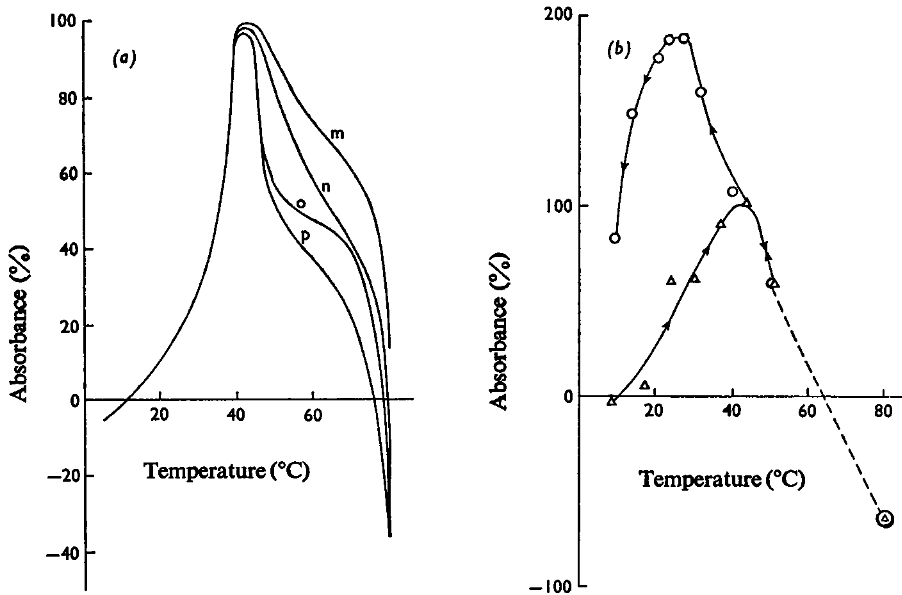


Fig. 3. *Effect of temperature on insoluble cytochrome c*

(*a*) The percentage change of absorbance with temperature for cytochrome *c* insolubilized on to either 2% agarose gel (line 'm' is the profile at 695nm, and 'n' those at 670 and 730nm) or 6% agarose gel (line 'o' is the profile at 695nm, and 'p' those at 670 and 730nm). (*b*) The effect of heating (△) and cooling (○) of cytochrome *c* insolubilized on to 2% agarose gel. The figures were drawn by arbitrarily giving a value of 100% to the peak absorbances during heating and 0% to the absorbances at 10°C. The cytochrome *c* solutions were prepared in distilled water and [cytochrome *c*] = 5.49mg/ml for the 6% gel and 3.39mg/ml for the 2% gel; path length of the cell was 1cm.

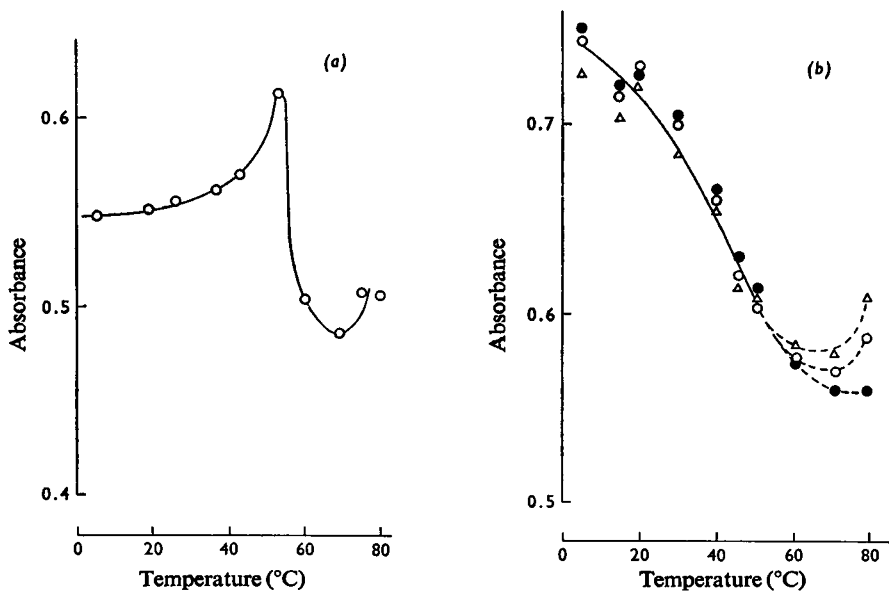


Fig. 4. *Temperature profiles of the control experiments*

(*a*) Temperature profile at 530nm of cytochrome *c* insolubilized on to 4% agarose gel. [Cytochrome *c*] = 20μM. (*b*) Temperature profile of a thick suspension of 6% agarose gel at 660nm (●), 695nm (○) and 730nm (△). All suspensions were prepared in distilled water.

although this effect is partly abolished by adding 3 M-NaCl.

Discussion

The 695 nm band of ferricytochrome *c* is very sensitive to conformational changes within the molecule and can be shown to disappear with increasing denaturation of the molecule, with the binding of ligands and on reduction of ferricytochrome *c* (Schejter & George, 1964; Greenwood & Palmer, 1965; Stellwagen, 1968). This conformational change is of a limited nature (Greenwood & Wilson, 1971), and the band is bleached completely before any major changes of conformation occur (Rupley, 1964). The insolubilization reaction results in a species of ferricytochrome *c* which retains the 695 nm band of the soluble species to a large extent, and therefore can be considered to be in the same conformational state as the soluble species.

The triazine method of attachment will certainly bind the gel to several of the surface lysines on cytochrome *c*, and this is interesting with respect to lysine-13, implicated in the binding of cytochrome oxidase (Takano *et al.*, 1972) and which extends across the crevice, and lysines 22, 25 and 100 which are displaced on reduction of ferricytochrome *c*, as the extended hairpin of residues 18–28 slides up to block the right channel. Strain on these lysines, all implicated in the redox changes, may affect the haem environment to a small extent and, although in either redox state the spectral features of insoluble cytochrome *c* are identical with those of the soluble species, this may account for the decrease in size of the conformationally more sensitive 695 nm band. The results from the optimizations certainly agree with this view as ΔS^0 is decreased in value on insolubilization, indicating a more restricted conformation in the region of methionine-80. As ΔH^0 is the same for soluble and insoluble ferricytochrome *c*, methionine-80 must still be the sixth ligand to the iron in insoluble ferricytochrome *c*.

Ferricytochrome *c* is more stable at high temperatures when insolubilized, and this is reflected as the regions termed 1 and 2 in the temperature profiles. The rigidity of agarose gel is caused by hydrogen-bonding, so there is more freedom of movement for the gel chains at higher temperatures, and less constraint on the cytochrome *c* molecules (i.e. region 3), which rapidly revert to the state they would have reached steadily if the constraint had been less at all temperatures. The latter situation was observed in the presence of 3.0 M-NaCl and was reflected in the higher value for ΔS^0 in these conditions. At room temperature the 695 nm band of insoluble ferricytochrome *c* is unaffected by high-NaCl conditions.

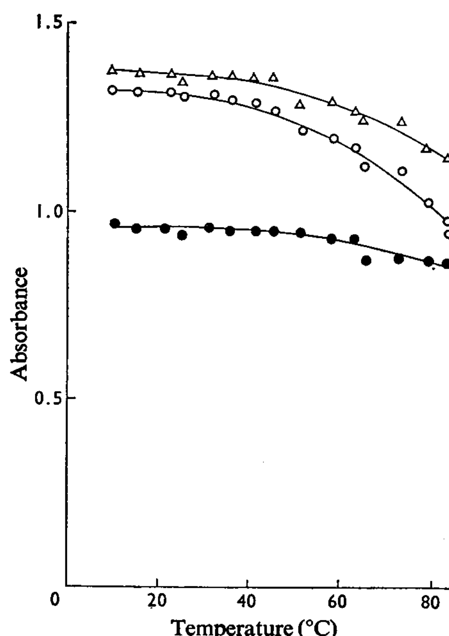


Fig. 5. *Effect of temperature on cytochrome c, insolubilized on to a 2% agarose gel suspension in* 3.0 M-*NaCl*

[Cytochrome *c*] = 1.03 mg/ml. ●, Absorbance at 660 nm; ○, 695 nm; △, 730 nm.

Table 4. *Optimized values of* ΔH^0 *and* ΔS^0 *for insoluble cytochrome c*

The experimental results were obtained by the method described in the text. The cytochrome *c* suspensions were prepared in distilled water. Values for the successful first and reflected optimizations are given.

	No. of experimental points	ΔH^0	ΔS^0	$\Delta\lambda$ (nm)
Insoluble cytochrome *c* (2% gel)	70	56.31	0.1494	Pooled results
Insoluble cytochrome *c* (6% gel)	55	60.20	0.1540	Pooled results
		59.32	0.1460	
Insoluble cytochrome *c* (2% gel) +3.0 M-NaCl	15	57.40	0.1612	35

These results parallel those for the soluble cytochrome *c*–phosvitin complex investigated by Taborsky (1970).

The method outlined above can be applied easily to any particulate system where there is an isolated spectrophotometric change and where an equation can be derived for the fractional saturation (y). More complex systems can be analysed in two ways, first, by a more complex derivation (see eqn. 6), or, secondly, by application of the simple equation at different values of $\Delta\lambda$, and extrapolating the optimized parameters to $\Delta\lambda = 0$. The applicability of each of these methods depends on the system under inspection.

We thank Dr. Selwyn for the use of his split-beam spectrophotometer, and Dr. Ottaway for help with computing. T. A. M. acknowledges an M.R.C. grant for part of this work.

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